Carbohydrate Research, 141 (1985) 111–119
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

STRUCTURAL CHARACTERISATION OF A NEUTRAL ANTITUMOUR β-D-GLUCAN EXTRACTED WITH HOT SODIUM HYDROXIDE FROM CULTURED FRUIT BODIES OF *Grifola frondosa*

KAZUYOSHI IINO, NAOHITO OHNO, IWAO SUZUKI, TOSHIO MIYAZAKI, TOSHIRO YADOMAE,
Laboratory of Immunopharmacology of Microbial Products, Tokyo College of Pharmacy, Horinouchi, Hachioji, Tokyo 192-03 (Japan)

SHOZO OIKAWA, AND KICHIRO SATO
Nippon Beet Sugar Mfg. Co., Ltd., Kyobashi 2-3-13, Chuo-ku, Tokyo 104 (Japan)
(Received October 15th, 1984; accepted for publication, February 4th, 1985)

ABSTRACT

The structural characterisation of an antitumour β-D-glucan (grifolan-7N), obtained from the hot sodium hydroxide extract of *Grifola frandosa*, is described. Grifolan-7N, purified by digestion with alpha-amylase, precipitation with ethanol, and chromatography on Con A–Sepharose, gave a single and symmetrical peak on gel filtration with Sepharose CL-4B (0.2M NaOH/8M urea) and had a molecular weight of ~1,200,000. The results of methylation analysis, ^{13}C-n.m.r. spectroscopy, Smith degradation, and enzymic digestion indicated grifolan-7N to be a (1→3)-linked β-D-glucan having a single β-D-glucopyranosyl group attached to position 6 of almost every third backbone unit. Grifolan-7N showed potent activity against the solid Sarcoma 180 in mice.

INTRODUCTION

Hot-water extracts of many kinds of mushrooms have long been used traditionally in Japan and China for cancer therapy. It has been suggested that the antitumour activity of the polysaccharides extracted from the cultured fruit bodies of Basidiomycetes (*e.g.*, lentinan[1] and PS-K[2]) is due to activation of the immune system, and some of these polysaccharides are now used clinically.

Grifola frondosa (which belongs to the Basidiomycetes, Aphyllophorales, Polypolaceae) is an edible mushroom, and the polysaccharide fractions extracted from the cultured fruit bodies with hot water and cold and hot aqueous sodium hydroxide showed potent antitumour activity[3]. These fractions contained neutral and acidic antitumour glucans[4] and it was concluded that the antitumour activity was due to a 6-branched (1→3)-β-D-glucan. Also, a polysaccharide fraction (GF-1), obtained by the conventional copper complex method from the hot-water extract[5],

0008-6215/85/$ 03.30

showed potent antitumour activity on murine syngeneic systems such as Meth-A-BALB/c and MM-46-C3H/He.

We now report the structural characterisation of the neutral antitumour glucan (grifolan-7N) extracted from the fruit body of *G. frondosa* with hot sodium hydroxide.

EXPERIMENTAL

Materials. — Sepharose CL-4B and ConA–Sepharose were obtained from Pharmacia, Bio-gel P-2 (50–100 mesh) was from Bio-Rad, and exo-(1→3)-β-D-glucanase (Lysing Enzymes, No. L-8757; from Basidiomycetes, *Rhizoctonia solani*) and alpha-amylase (No. A-6380) were obtained from Sigma.

General methods. — T.l.c. was performed on cellulose (Merck, 5577), using ethyl acetate–pyridine–acetic acid–water (5:5:1:3) and detection with alkaline silver nitrate. Alditols and methylated sugars were converted into the corresponding alditol acetates and subjected to g.l.c., using a Shimadzu GC-6A instrument equipped with a flame-ionisation detector. H.p.l.c. was performed using a column of silica-NH_2. Optical rotations were measured with a DIP-4 (JASCO) instrument equipped with a 5-cm quartz cell, and a concentration of 1 mg/mL. Sugar contents were determined as "anhydroglucose" by the phenol–sulfuric acid method, using D-glucose as standard[6]. Protein contents were determined by the Lowry–Folin method with bovine serum albumin as the reference[7].

Extraction with hot alkali. — The cultured fruit body of *Grifola frondosa* was defatted with aqueous 80% ethanol, and then extracted successively with hot water and cold sodium hydroxide. The residue was extracted at 65° with aqueous 10% sodium hydroxide containing 5% of urea. The extract was neutralised with acetic acid, and then dialysed against tap water for 2 days and distilled water for 1 day. The non-dialysable fraction was centrifuged, and the supernatant solution was concentrated and precipitated with 4 vol. of ethanol. The precipitate (F-7, 8%) was collected, and dried with acetone and then ether. Solutions of F-7 (0.3 g) in 2M urea (15 mL) were added to the tops of columns (3 × 10 cm) of DEAE-Sephadex A-25 (HCO_3^-). Each column was eluted with 2M urea, the appropriate fraction was dialysed, and the non-dialysable fraction was collected (F-7N, 55.1%). The combined F-7N fractions (1 g) were dissolved in 50mM Tris-HCl buffer (pH 6.9, 700 mL) and digested with alpha-amylase (20 mg) at 37° for 24 h. The reaction was terminated by heating at 100° for 5 min, and the mixture was dialysed against water. The non-dialysable fraction was concentrated and lyophilised. A portion (200 mg) of the resulting material (432 mg) was dissolved in distilled water (20 mL) and precipitated by adding 1 vol. of ethanol. A portion (40 mg) of the precipitate (149 mg) was dissolved in 0.02M Tris-HCl buffer (pH 7.4), added to the top of a column (3 × 10 cm) of ConA–Sepharose, and eluted with the same buffer. The appropriate fraction was dialysed against water, and the non-dialysable fraction was concentrated and lyophilised to give grifolan-7N (27 mg).

Gel filtration. — The d.p. of the enzymic hydrolysate was determined by elution from a column (2.2 × 146 cm) of Bio-gel P-2 with water. The carbohydrate and protein contents of each fraction (3.7 mL) were determined.

Methylation analysis. — Methylation was performed by the method of Hakomori[8]. The fully methylated product was treated with aqueous 90% HCOOH (1 mL) at 100 ° for 10 h in a sealed tube. After evaporation of the formic acid, the residue was hydrolysed with M trifluoroacetic acid at 100° for 5 h and the hydrolysate was concentrated to dryness. The resulting partially methylated sugars were reduced with sodium borohydride for 2 h and the resulting alditols were treated with pyridine–acetic anhydride at 100° for 1 h. G.l.c. of the partially methylated alditol acetates was performed with a glass column (200 × 0.3 cm) packed with 0.3% of OV-275/0.4% of XF-1150, and a temperature programme of 120°→190° at 2°/min. The molar ratio of each methylated derivative was calculated by reference to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol as 1.0.

N.m.r. studies. — ^{13}C-N.m.r. spectra (50.1 MHz) were recorded at 60° for solutions in Me_2SO-d_6, or at room temperature for solutions in D_2O, with a JEOL FX-200 spectrometer. The spectra were obtained in the pulsed Fourier-transform mode with proton decoupling.

Periodate oxidation and Smith degradation. — Grifolan-7N (12 mg) was oxidised with 10mM sodium metaperiodate (40 mL) at 4° in the dark, and periodate consumption was monitored by the method of Avigad[9]. When the reaction was complete (173 h), excess of periodate was reduced with ethylene glycol, and the mixture was dialysed against tap water for 2 days and distilled water for 1 day. The non-dialysable fraction was reduced with sodium borohydride (50 mg) at 4° for 48 h. After acidification with acetic acid, the mixture was dialysed and lyophilised to give grifolan-7N(I/B) (10.8 mg).

A portion (1.37 mg) of the polyalcohol was hydrolysed with M trifluoroacetic acid (1 mL) at 100° for 5 h. The resulting monosaccharide mixture was analysed by g.l.c. as the alditol acetates.

Another portion (1.71 mg) of the polyalcohol was partially hydrolysed with 0.05M sulfuric acid (2.0 mL) at 25° for 75 h. The mixture, after neutralisation, was dialysed against distilled water for 2 days. The dialysable fraction was concentrated, and then de-ionised by using columns of Amberlite CG-4B (HO^-) and Dowex-50 (H^+) resins. The products were converted into alditol acetates and analysed by g.l.c. The non-dialysable fraction was methylated, and then analysed by g.l.c.

Enzymic hydrolysis. — Grifolan-7N (21.19 mg) was dissolved in 0.03M McIlvaine buffer (pH 4.9, 20 mL), and exo-(1→3)-β-D-glucanase (12.13 mg, 10 mL) was added. The mixture was shaken at 37° for 25 h, and the reaction was terminated by heating at 100° for 15 min. The resulting solution was dialysed against distilled water for 2 days, and the dialysable and non-dialysable fractions were collected. The dialysable fraction was analysed by t.l.c., h.p.l.c., and gel filtration.

Assay of antitumour activity. — Male ICR mice (6 weeks old and weighing 27–30 g) were obtained from Shizuoka Agricultural Cooperative Association for

Laboratory Animals. Sarcoma 180 tumour ascites cells were kindly supplied by Dr. T. Sasaki (National Cancer Research Institute, Japan) and were maintained by weekly passage in ICR mice. Tumour cells (5×10^6) were inoculated subcutaneously into the right groin of mice. Grifolan-7N was administered for 10 consecutive days from 24 h after the inoculation of the tumour, and the mice were sacrificed 5 weeks later. The inhibition ratio was

$$\left(1 - \frac{\text{average tumour weight of the treated group}}{\text{average tumour weight of the control group}}\right) \times 100(\%)$$

Complex formation with Congo Red. — The change of absorption of Congo Red (Wako Pure Chemical Co.) in the presence of glucans was performed by the procedure of Ogawa *et al.*[10]. Glucan solutions (1 mg/mL) and 2.16×10^{-4}M Congo Red were mixed in equal volumes, and λ_{max} was measured by using an Hitachi 557 spectrophotometer.

RESULTS AND DISCUSSION

An antitumour β-D-glucan extracted by hot alkali from the fruit bodies of *Grifola frondosa* was purified by application in sequence of digestion with alpha-amylase, precipitation with ethanol, and chromatography on ConA–Sepharose. The glucan (grifolan-7N) showed potent activity against the Sarcoma 180 solid tumour after i.p. administration (100 μg/mouse for 10 days caused 97% inhibition with complete regression in 4 out of 10 mice; Table I).

Grifolan-7N showed a single and symmetrical peak on elution from Sepharose CL-4B (0.2M NaOH/8M urea, Fig. 1). The K_{av} value indicated a molecular weight of ~1,200,000. The glucan had $[\alpha]_D$ +1.8° (*c* 0.1, water). Methylation analysis of the glucan gave 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol, and 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-glucitol in the molar ratios 1.0:1.96:1.07 (Table II). The glucan also

TABLE I

ANTITUMOUR EFFECT ON SOLID SARCOMA 180[a]

Sample	*Dose × 10 (μg/mouse)*	*Tumour weight (g, mean ±s.d.)*	*Inhibition[b] ratio (%)*	*Complete regression[b]*	*Significance[c]*
Grifolan-7N	4	3.39 ±2.30	9.4	0/8	n.s.[d]
	20	0.90 ±1.59	75.9	2/10	$p < 0.01$
	100	0.08 ±0.12	97.9	4/10	$p < 0.001$
Nil(saline)		3.74 ±2.60	—	0/12	—

[a]Sarcoma 180 tumour cells (5×10^6) were inoculated subcutaneously. Each sample was administered i.p. as a solution in saline. [b]Determined 35 days after inoculation of the tumour. [c]Evaluated according to Student's t-test; $p < 0.05$ was taken as the criterion of a significant difference. [d]Not significant.

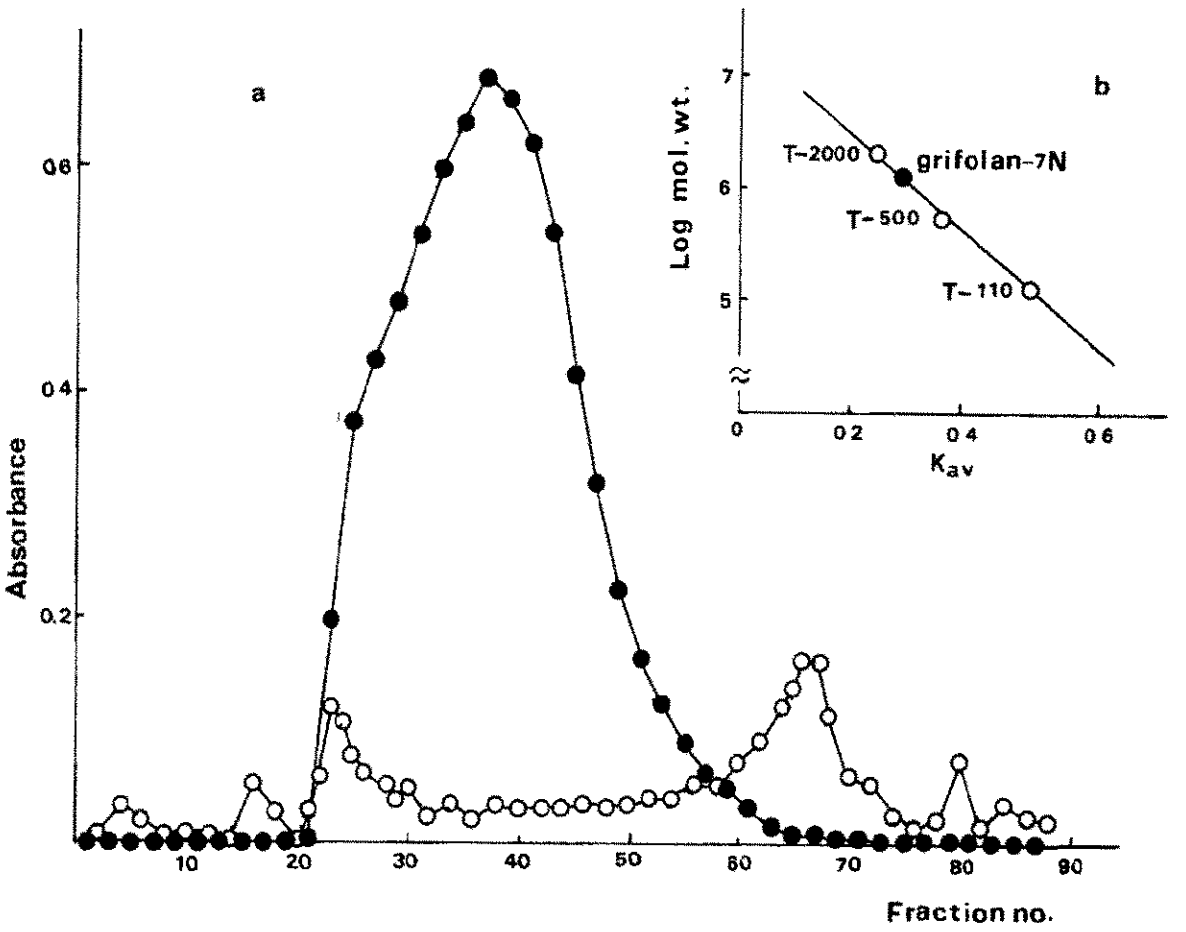

Fig. 1. (*a*) Gel filtration of grifolan-7N (50 mg) on a column (1.8 × 90 cm) of Sepharose CL-4B on elution with 0.2M NaOH/8M urea. Fractions (2.2 mL) were assayed for carbohydrate (—●—) and protein (—○—). (*b*) Determination of molecular weight of grifolan-7N by gel filtration on Sepharose CL-4B.

yielded small quantities of other partially methylated compounds, which probably arose from contaminants because their proportions varied. The ^{13}C-n.m.r. spectrum (Fig. 2) of a solution of the glucan in Me_2SO-d_6 showed signals similar to those of scleroglucan[11], which is a branched (1→3)-β-D-glucan. These results indicate grifolan-7N to contain 3-substituted, 3,6-disubstituted, and non-reducing terminal β-D-glucose residues in the molar ratios 2:1:1.

Grifolan-7N consumed 0.54 mol of periodate per D-glucosyl residue.

TABLE II

G.L.C. OF ALDITOL ACETATES DERIVED FROM THE METHYLATED POLYSACCHARIDES

Alditol acetate	*Grifolan-7N*	*Grifolan-7N(I/B)*	*Partially hydrolysed grifolan-7N(I/B)*
2,3,4,6-Me_4-Glc	1.0	—	—
2,4,6-Me_3-Glc	1.96	1.00	1.00
2,3,4-Me_3-Glc	0.05	—	—
2,3,6-Me_3-Glc	—	—	—
2,6-Me_2-Glc	0.09	—	—
3,4,6-Me_3-Glc	0.07	—	—
2,4-Me_2-Glc	1.07	0.58	0.09
2,3-Me_2-Glc	—	—	—
3,6-Me_2-Glc	—	—	—

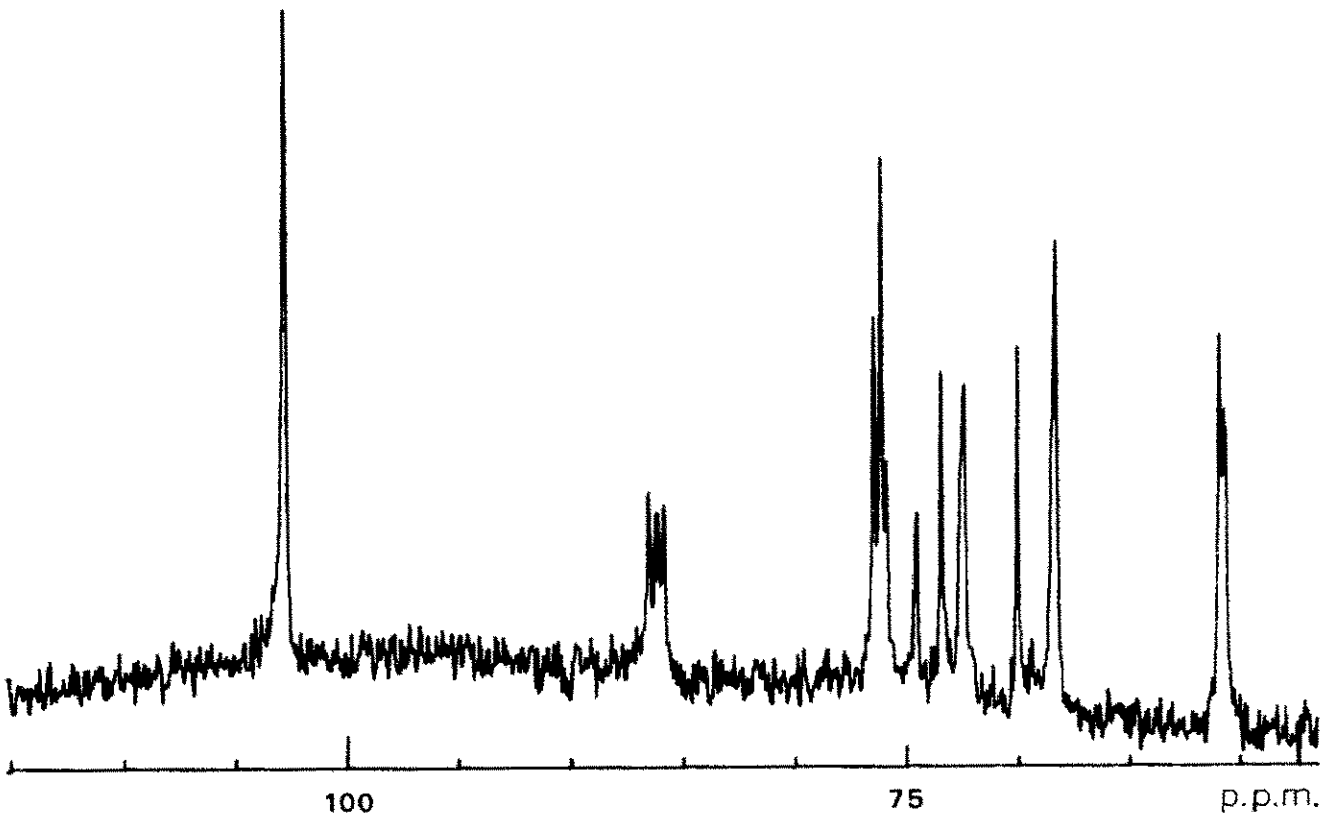

Fig. 2. ^{13}C-N.m.r. spectrum of grifolan-7N in Me_2SO-d_6 at 60°.

Borohydride reduction of the product and complete hydrolysis of the resulting polyalcohol gave D-glucitol and glycerol. On methylation analysis, the polyalcohol gave 2,4,6-tri- and 2,4-di-*O*-methylglucose, in the molar ratio 1.00:0.58. This ratio was consistent with that of the native glucan.

Mild, acid hydrolysis (0.05M H_2SO_4, 25°, 72 h) of the polyalcohol gave glycerol and D-glucitol (trace) in the dialysable fraction. Methylation analysis of the non-dialysable fraction gave 2,4,6-tri- and 2,4-di-*O*-methyl-D-glucitol in the molar ratio 1.00:0.09; 2,3,4,6-tetra-*O*-methyl-D-glucose was not detected. These findings suggest that the main chain of the β-D-glucan was (1→3)-linked with β-D-glucosyl groups attached at position 6 of some (1 in 3 on average) of the main-chain units. This structure is similar to those of other polysaccharides obtained from Basidiomycetes and Ascomycetes (*e.g.*, lentinan[1,12], scleroglucan[11,13], and schizophyllan[14]). This primary structure is also similar to the glucans extracted from *G. frondosa* with zinc chloride and sodium hydroxide[15,16].

When grifolan-7N was treated with exo-(1→3)-β-D-glucanase obtained from *Rhizoctonia solani*[17], ~40% was digested, and recovered from the dialysable fraction as gentiobiose and glucose (t.l.c., h.p.l.c., gel filtration). Methylation analysis of the non-dialysable fraction revealed that the proportion of 2,4,6-tri-*O*-methyl derivative was slightly diminished compared to that for the parent glucan (molar ratios of tetra-, tri-, and di-*O*-methyl derivatives, 1:1.59:0.98). Although the mode of action of this enzyme is not known precisely, the results suggest that the glucan contains some resistant linkages which, according to the methylation data, could be located at the branch points.

Under physiological conditions, (1→3)-β-D-glucans adopt a helical conforma-

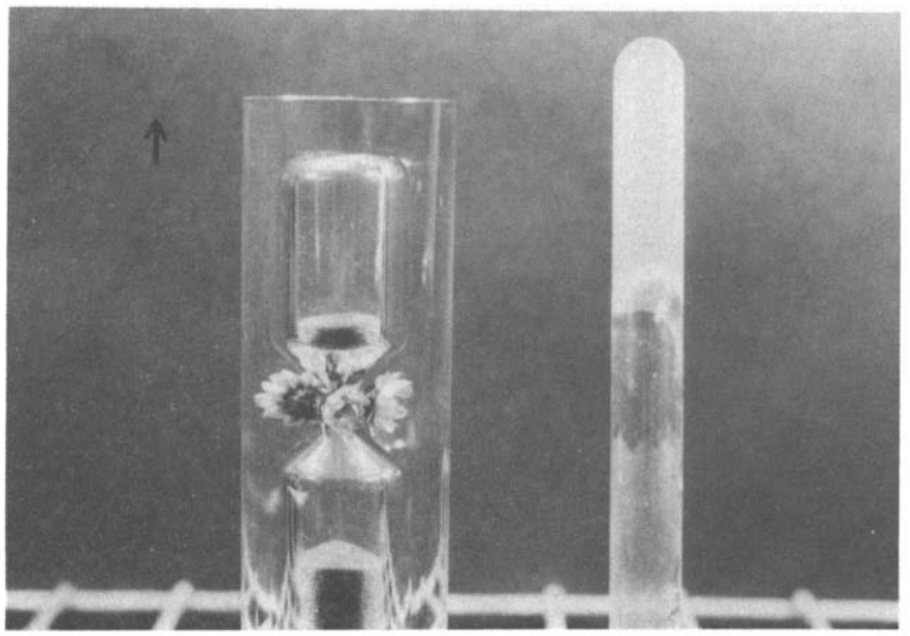

Fig. 3. Gel formation of grifolan-7N at neutral pH (20 mg/mL, aqueous solution).

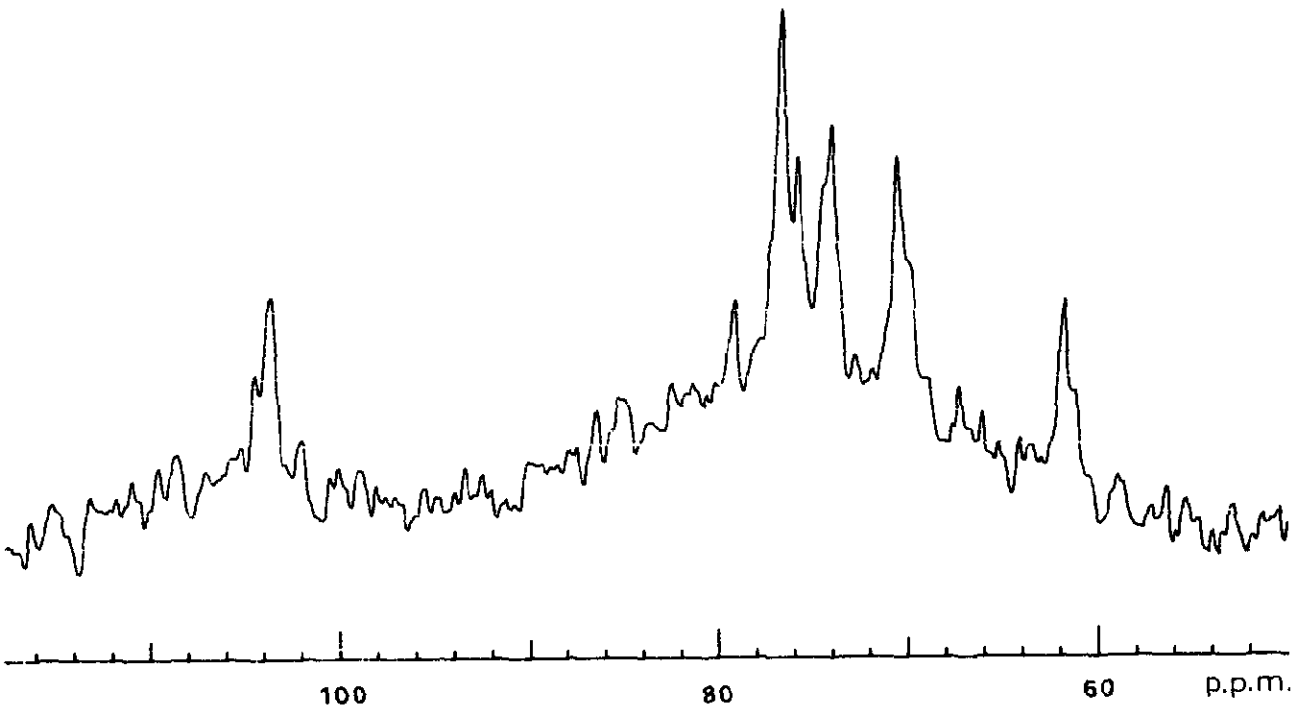

Fig. 4. ^{13}C-N.m.r. spectrum of grifolan-7N in D_2O at room temperature.

tion[18] which affects the $[\alpha]_D$ value, the n.m.r. spectrum, and the gel-filtration properties, *etc.* Grifolan-7N formed a soft gel at neutral pH (Fig. 3). It induced metachromasy with Congo Red, as do helix-forming glucans (such as curdlan) below 0.3M NaOH (Table III). However, since the effect occurred below 0.2M NaOH, the helical conformation of grifolan-7N is probably weaker than that of curdlan. The $[\alpha]_D$ values of helix-forming glucans are affected by variation in the concentration of urea or sodium hydroxide, but they were scarcely changed for grifolan-7N (Table IV). Solutions in D_2O of glucans that form tight helixes show no ^{13}C-n.m.r. signals, in contrast to grifolan-7N (Fig. 4) whose ^{13}C-n.m.r. spectrum is similar to that of lentinan [an antitumour (1→3)-β-D-glucan[19]]. Hence, it is suggested that grifolan-7N forms a weakly rigid, helical conformation under the physiological con-

TABLE III

ABSORPTION MAXIMUM OF CONGO RED IN ALKALINE SOLUTION IN THE PRESENCE AND IN THE ABSENCE OF POLYSACCHARIDES

Sample	λ_{max} *(nm)*		
	0.1M NaOH	*0.2M NaOH*	*0.3M NaOH*
Congo Red only	482	479	480
Congo Red + amylopectin	482	479	481
Congo Red + islandican	479	480	480
Congo Red + laminaran	481	478	n.d.[a]
Congo Red + curdlan	500	488	480
Congo Red + grifolan-7N	496	479	480

[a]Not determined.

TABLE IV

$[\alpha]_D$ VALUES OF GRIFOLAN-7N

Water	+1.8°				
Urea		*Urea*		*NaOH*	
M	+1.3°	5M	+0.6°	0.1M	+1.5°
2M	+1.6°	6M	−1.2°	0.2M	+2.3°
3M	+0.8°	7M	+0.3°	0.3M	+0.4°
4M	±0°	8M	±0°	0.4M	+0.6°

ditions. The rigidity of the helix would depend on the distribution of the branch points which, on the basis of the results of digestion with exo-(1→3)-β-D-glucanase, is probably irregular.

Shida *et al.*[20] reported that the skeletal part of *Lentinus edodes* contained three structurally different glucans. The outermost part seemed to contain mainly (1→3)- and (1→6)-β-D-glucosidic linkages and, structurally, was closely similar to lentinan, a water-soluble β-D-glucan from *L. edodes*. The middle part appeared to contain mainly (1→6)-β-D-glucosidic linkages, and the innermost part contained (1→6)- and (1→3)-β-D-glucosidic linkages. Thus, grifolan-7N is a lentinan-type glucan and probably exists in the outermost skeletal part of the mushroom *G. frondosa*. This antitumour glucan is being investigated further.

ACKNOWLEDGMENTS

We thank Mr. T. Takeyama, Mr. T. Kinoshita, Mr. J. Saito, and Mr. M. Matsumura for technical assistance.

REFERENCES

1 G. CHIHARA, Y. MAEDA, J. HAMURO, T. SASAKI, AND F. FUKUOKA, *Nature (London)*, 222 (1969) 687–688.
2 S. TSUKAGOSHI AND F. OHASHI, *Gann*, 65 (1974) 557–558.
3 N. OHNO, I. SUZUKI, S. OIKAWA, K. SATO, T. MIYAZAKI, AND T. YADOMAE, *Chem. Pharm. Bull.*, 32 (1984) 1142–1151.
4 N. OHNO, K. IINO, I. SUZUKI, S. OIKAWA, K. SATO, T. MIYAZAKI, AND T. YADOMAE, *Chem. Pharm. Bull.*, 33 (1985) 1181–1186.
5 I. SUZUKI, T. ITANI, N. OHNO, S. OIKAWA, K. SATO, T. MIYAZAKI, AND T. YADOMAE, *J. Pharm. Dyn.*, 7 (1984) 492–500.
6 M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH, *Anal. Chem.*, 28 (1956) 350– 356.
7 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265–275.
8 S. HAKOMORI, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
9 G. AVIGAD, *Carbohydr. Res.*, 11 (1969) 119–123.
10 K. OGAWA, J. TSURUGI, AND T. WATANABE, *Chem. Lett.*, (1972) 689–692.
11 M. RINAUDO AND M. VINCENDON, *Carbohydr. Polymers*, 2 (1982) 135–144.
12 G. CHIHARA, J. HAMURO, Y. MAEDA, Y. ARAI, AND F. FUKUOKA, *Cancer Res.*, 30 (1970) 2776–2781.
13 P. P. SINGH, R. L. WHISTLER, R. TOKUZEN, AND W. NAKAHARA, *Carbohydr. Res.*, 37 (1974) 245–247.
14 N. KOMATSU, S. OHKUBO, S. KIKUMOTO, K. KIMURA, G. SATO, AND S. SASAKI, *Gann*, 60 (1969) 137–144.
15 K. KATO, T. INAGAKI, H. SHIBAGAKI, R. YAMAUCHI, K. OKUDA, T. SANO, AND Y. UENO, *Carbohydr. Res.*, 123 (1983) 259–265.
16 K. KATO, T. INAGAKI, T. TERANISHI, R. YAMAUCHI, K. OKUDA, T. SANO, AND Y. UENO, *Carbohydr. Res.*, 124 (1983) 247–252.
17 S. UKAI, C. HARA, AND T. KIHO, *Chem. Pharm. Bull.*, 30 (1980) 2147–2154.
18 T. NORISUYE, T. YANAKI, AND H. FUJITA, *J. Polym. Sci.*, 18 (1980) 547–558.
19 H. SATO, T. OHKI, N. TAKASUKA, AND T. SASAKI, *Carbohydr. Res.*, 58 (1977) 293–305.
20 M. SHIDA, Y. USHIODA, T. NAKAJIMA, AND K. MATSUDA, *J. Biochem. (Tokyo)*, 90 (1981) 1093–1100.